Chiral Recognition of Ferrocytochrome c(II) in Electron-transfer Reactions with Cobalt(III) Complexes

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Stereoselective electron-transfer reactions were carried out between ferrocytochrome c, cyt c(II) and the cobalt(III) complexes tris(2,2′-bipyridine)cobalt(III), $[Co(bipy)_3]^{3+}$ and tris(pentane-2,4-dionato)cobalt(III), $[Co(pd)_3]$. The reduction of $[Co(bipy)_3]^{3+}$ proceeds much more rapidly than that of $[Co(pd)_3]$. At the same time, the stereoselectivity for the former is much greater than for the latter. These results are interpreted in terms of incorporation of bipy into a hydrophobic crevice of cyt c(II). The stereoselectivity exhibits interesting dependences on the reaction conditions such as the ethanol content in the solvent, pH and temperature. These dependences are discussed in terms of the closeness of approach of the cobalt(III) complexes to the chiral groups of cyt c(II).

Electron-transfer reactions between haemoproteins and transition-metal complexes have been studied extensively in the last decade,[1] in an attempt to elucidate the reaction mechanism, biological functions and reaction properties of haemoproteins. For instance, many kinetic studies of the oxidation-reduction reactions of haemoproteins,[2] and more recently of modified proteins,[3] have been reported. Surprisingly, however, very little is known about the stereoselectivity in electron-transfer reactions of haemoproteins except for only a few pioneering works,[4,5] whereas high stereoselectivity is a characteristic feature of biological reactions.[6] Stereoselectivity would be determined by the distance between the chiral groups and the substrate, whereas the reaction rate and oxidation–reduction potential of haemoproteins mainly depend on the situation of the active site and the haem part. Thus, the stereoselectivity is expected to offer new information about the reaction properties of haemoproteins.

In this work electron-transfer reactions between ferrocytochrome c, cyt c(II) and the complexes tris(pentane-2,4-dionato)cobalt(III), $[Co(pd)_3]$ and tris(2,2′-bipyridine)cobalt(III), $[Co(bipy)_3]^{3+}$, were investigated. Both reactions proceed stereoselectively. The stereoselectivity observed depends on the reaction conditions such as pH, temperature and ethanol content in the solvent. It also exhibits a very interesting difference between $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$, which is discussed in terms of incorporation of the bipy ligand into the haem crevice.

Experimental

Ferrocytochrome c was prepared by dithionite reduction of commercially available horse heart cytochrome c (Sigma type VI), and purified by gel filtration (Sephadex G50, 10 mm i.d. × 450 mm).[7] All these treatments were carried out at 4 °C and the cyt c(II) solution was stored under an argon atmosphere. The complex $[Co(pd)_3]$ was purchased from Nakarai Chemical Co. (extra-pure grade) and used after recrystallization from benzene and light petroleum (b.p. 30–60 °C). The salt $[Co(bipy)_3][ClO_4]_3$ was prepared according to a previous method,[8] and recrystallized from hot water. Its purity was confirmed by elemental analysis.

Typically, the electron-transfer reaction between cyt c(II) (1.0×10^{-4} mol dm^{-3}) and the cobalt(III) complex (2.5×10^{-3} mol dm^{-3}) was performed at 25 °C in 0–40% (v/v) ethanol–water with ionic strength (I) adjusted to 0.1 mol dm^{-3} (KCl) and phosphate buffer for pH 6.0–7.0 and acetate buffer for pH 4.0–5.0.* In the case of $[Co(pd)_3]$ reduction, the reaction solution was evaporated to dryness when the reaction was complete (48 h), and then the remaining $[Co(pd)_3]$ was dissolved in ethanol. The circular dichroism (CD) spectrum of the remaining $[Co(pd)_3]$ solution obtained above was measured with a JASCO J-500C spectropolarimeter. The insolubility of cyt c in ethanol was certified as follows. A solution containing cyt c(II) and cyt c(III) was treated in the same way as above but the resultant ethanol solution exhibited no CD absorption band in the range 500–700 nm in which $[Co(pd)_3]$ exhibits CD spectra. In the case of $[Co(bipy)_3]^{3+}$ reduction, a different treatment was applied because this complex is insoluble in ethanol. After completion of the reaction (2 h), the reaction solution was diluted by addition of 50 times its volume of water, and then its CD spectrum was measured. Because the obtained CD spectra include bands due to $[Co(bipy)_3]^{3+}$ and cyt c(III), the CD absorption of the remaining $[Co(bipy)_3]^{3+}$ was estimated by subtracting the CD absorption of cyt c(III) from the total CD absorption, the concentration of cyt c(III) being determined spectrophotometrically. The stereoselectivity was defined by the enantiomer excess (e.e., in % based on the cobalt complex consumed) which was determined from the CD absorption band of the remaining cobalt(III) complex.

Kinetic runs were performed with 2.0×10^{-5} mol dm^{-3} of cyt c(II) and 8.0×10^{-4}–1.4×10^{-3} mol dm^{-3} of the cobalt(III) complex, in which the decrease in cyt c(II) was followed spectrophotometrically. The observed first-order rate constant was analysed by Michaelis–Menten kinetics. The parameters, k_{et} and K_M, are defined as $k_2[\text{cyt c(II)}]_{t=0}$ and $(k_{-1} + k_{et})/k_1$.

$$\text{cyt c(II)} + \text{Co}^{III} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{cyt c(II)} \cdots \text{Co}^{III}] \qquad (1)$$

$$[\text{cyt c(II)} \cdots \text{Co}^{III}] \xrightarrow{k_2} \text{cyt c(III)} + \text{Co}^{II} \qquad (2)$$

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* The water before mixing with ethanol has these pH values. After mixing, the pH shifts to a slightly higher value: in phosphate or acetate buffer (I = 0.1 mol dm^{-3}), from 4.0 to 4.2, 5.0 to 5.2, 6.0 to 6.3 and 7.0 to 7.3 (15% v/v ethanol), from 7.0 to 7.4 and 4.0 to 4.3 (20% v/v ethanol), from 7.0 to 7.6 and 4.0 to 4.5 (30% v/v ethanol) and from 7.0 to 7.4 and 4.0 to 4.8 (40% v/v ethanol); in Tris-cacodylic acid (2-amino-2-hydroxymethylpropane-1,3-diol–methylarsinic acid) buffer (I = 0.1 mol dm^{-3}) from 6.7 to 6.9 (15% v/v ethanol) and to 7.0 (30% v/v ethanol).

Table 1 Comparison of stereoselectivity and reaction rate between the reductions of $[Co(bipy)_3]^{3+}$ and $[Co(pd)_3]^a$

Reaction conditions			$[Co(pd)_3]$		$[Co(bipy)_3]^{3+}$	
pH	Ethanol (% v/v)	Buffer	$k_{2,obs}/dm^3\ mol^{-1}\ s^{-1}$	e.e. (%)	$k_{2,obs}/dm^3\ mol^{-1}\ s^{-1}$	e.e. (%)
7.0	0	Phosphate	*b*	*b*	3.1×10^2	8.7 ± 0.3
6.7	0	Tris[c]	*b*	*b*	1.5×10^2	9.1 ± 0.2
7.0	20	Phosphate	2.8×10^{-2}	0.52 ± 0.02	5.8×10^2	8.1 ± 0.5
7.0	40	Phosphate	*d*	2.34 ± 0.07	*d*	12.1 ± 0.1
4.0	40	Acetate	*d*	5.34 ± 0.29	*d*	11.3 ± 0.3

[a] $I = 0.1$ mol dm^{-3} (KCl), 25 °C. [b] Unable to perform reaction because of insolubility of $[Co(pd)_3]$ in this solvent. [c] Tris–cacodylic acid. [d] Error is too large in kinetic experiments.

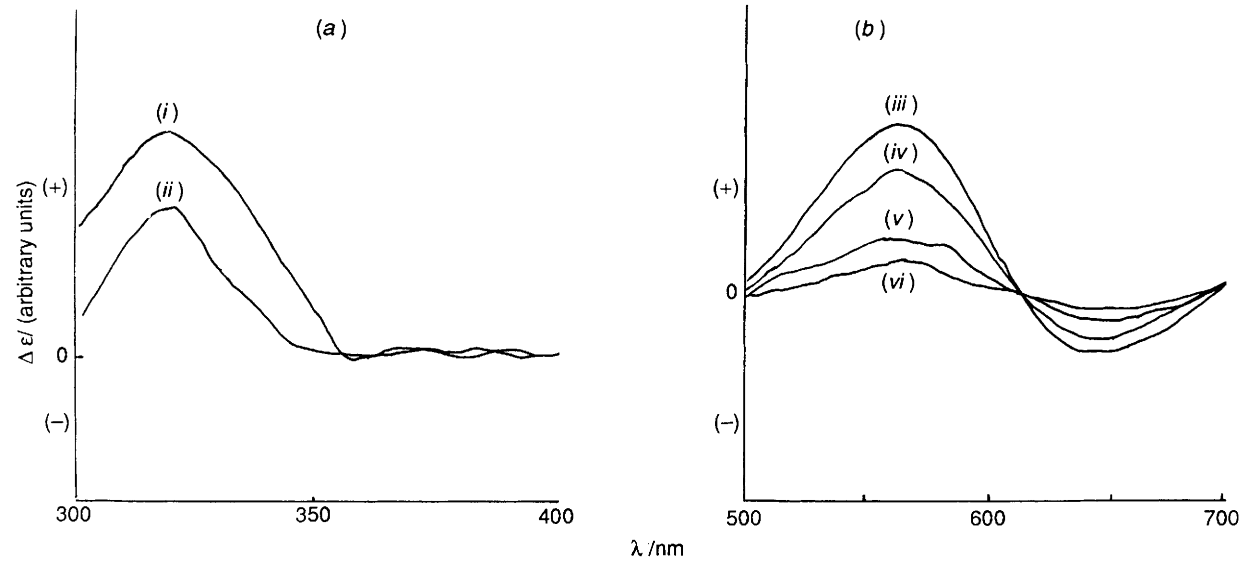

Fig. 1 CD spectra of the remaining $[Co(bipy)_3]^{3+}$ and $[Co(pd)_3]$ in the reduction at 25 °C and $I = 0.1$ mol dm^{-3} of (*a*) $[Co(bipy)_3]^{3+}$ reduction (pH 7.0, phosphate buffer) and (*b*) $[Co(pd)_3]$ (pH 4.0, acetate buffer). Ethanol content (% v/v): 40 (*i*) and (*iii*), 30 (*iv*), 20 (*v*), 15 (*vi*) and 0 (*ii*)

Results

Electron-transfer reactions of cyt c(II) with *rac*-$[Co(bipy)_3]^{3+}$ and *rac*-$[Co(pd)_3]$ occur easily. As shown by the second-order rate constant, $k_{2,obs}$, defined by $-d[\text{cyt c(II)}]/dt = k_{2,obs}[\text{cyt c(II)}][Co^{III}]$, given in Table 1, the reduction of $[Co(bipy)_3]^{3+}$ proceeds much more rapidly than that of $[Co(pd)_3]$. This is related to the greater ability of $[Co(bipy)_3]^{3+}$ to approach the active site of cyt c(II), as will be discussed later.* In the $[Co(bipy)_3]^{3+}$ reduction, CD spectra of the reaction solution exhibit a positive absorption around 320 nm, as shown in Fig. 1, which coincides well with that at 321 nm for Δ(+)-$[Co(bipy)_3]^{3+}$.[11a] This means that an excess of Δ(+)-$[Co(bipy)_3]^{3+}$ exists after the reaction and that cyt c(II) reduces Λ(−)-$[Co(bipy)_3]^{3+}$ more easily than the Δ(+) form.† In the $[Co(pd)_3]$ reduction, CD spectra of the reaction solution exhibit a positive absorption around 570 nm. This means that an excess of Λ(−)-$[Co(pd)_3]$ exists[11b] and cyt c(II) reduces Δ(+)-$[Co(pd)_3]$ more rapidly than the Λ(−) form. The stereoselectivity of the reduction of $[Co(bipy)_3]^{3+}$ is much larger than that of $[Co(pd)_3]$,[4b] as is clearly shown by the typical examples in Table 1.

The stereoselectivity observed is sensitive to the reaction conditions such as the ethanol content in the solvent, pH and temperature. In the case of $[Co(pd)_3]$, the stereoselectivity increases monotonously upon increasing the % (v/v) ethanol in phosphate buffer, as shown in Fig. 2, but no stereoselectivity is found in Tris–cacodylic acid buffer. On the other hand, reduction of $[Co(bipy)_3]^{3+}$ proceeds stereoselectively in both buffers, and the stereoselectivity depends on the ethanol content in a complicated manner. For instance, this dependence is quite different between phosphate and Tris–cacodylic acid buffers. In the phosphate buffer the stereoselectivity increases upon going from 0 to 10% ethanol, decreases upon going to 20% ethanol and again increases with a further increase to 40% ethanol. In Tris–cacodylic acid buffer the stereoselectivity increases upon going to 10% ethanol but then decreases monotonously with further increase in ethanol content. These differences in stereoselectivity between $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$ and between phosphate and Tris–cacodylic acid buffers suggest that the reaction properties of cyt c(II) are influenced by substrates and buffers.

The stereoselectivity dependence on pH is rather simple, compared to the dependence on ethanol content. As shown in Fig. 3, the stereoselectivity increases upon lowering the pH in the reduction of both $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$. This means that close approach of $[Co(bipy)_3]^{3+}$ to the chiral groups of cyt c(II) is influenced by pH in a similar way to that of $[Co(pd)_3]$. The reaction temperature is also an important factor in the stereoselectivity, as shown in Fig. 4. In the reduction of $[Co(pd)_3]$ the stereoselectivity suddenly decreases to ≈0 at 40 °C. With $[Co(bipy)_3]^{3+}$ the stereoselectivity at 40 °C is lower than that at 25 °C, but it is still considerably large, much larger than the stereoselectivity of $[Co(pd)_3]$ reduction at 25 °C. This is an interesting difference.

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* The redox potential is also an important factor. Although its value for $[Co(bipy)_3]^{3+}$ has been reported,[9] that of $[Co(pd)_3]$ cannot be measured because of the irreversibility of its reduction.[10] So, no discussion regarding the redox potential is made.

† The self-exchange reaction between $[Co(bipy)_3]^{3+}$ and $[Co(bipy)_3]^{2+}$ proceeds slowly.[9] However, a CD absorption band of the reaction solution does not change after several hours. Thus, the data obtained here are not influenced by the self-exchange reaction.

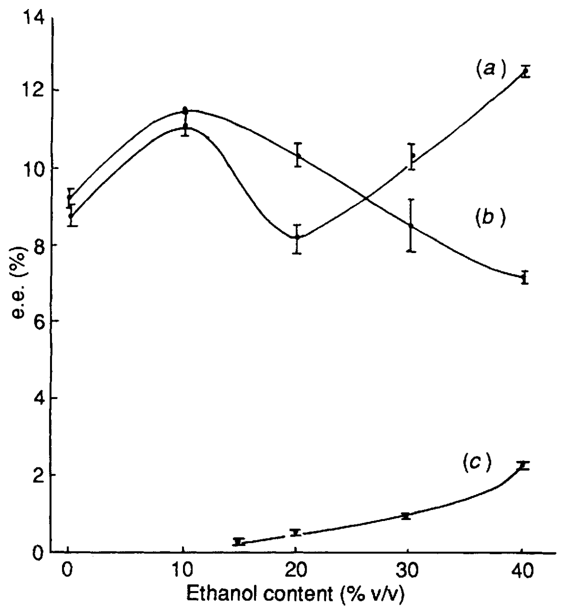

Fig. 2 Effect of ethanol content on the stereoselectivity of the electron-transfer reaction at 25 °C and $I = 0.1$ mol dm^{-3} between cyt c(II) and (*a*) $[Co(bipy)_3]^{3+}$ in phosphate buffer (pH 7.0), (*b*) $[Co(bipy)_3]^{3+}$ in Tris–cacodylic acid buffer (pH 6.7) and (*c*) $[Co(pd)_3]$ in phosphate buffer (pH 7.0)

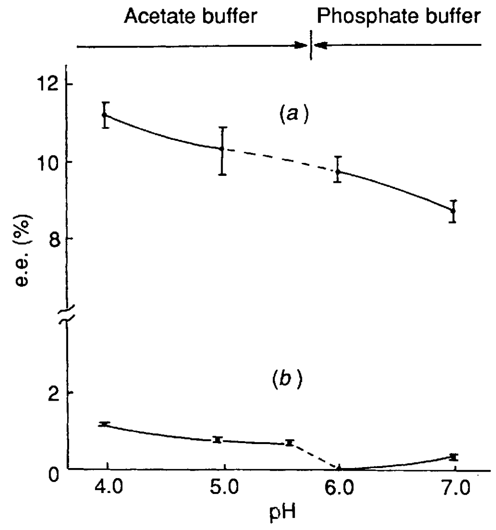

Fig. 3 Effect of pH on the stereoselectivity of the electron-transfer reaction at 25 °C and $I = 0.1$ mol dm^{-3} between cyt c(II) and (*a*) $[Co(bipy)_3]^{3+}$ (0% ethanol) and (*b*) $[Co(pd)_3]$ (15% ethanol)

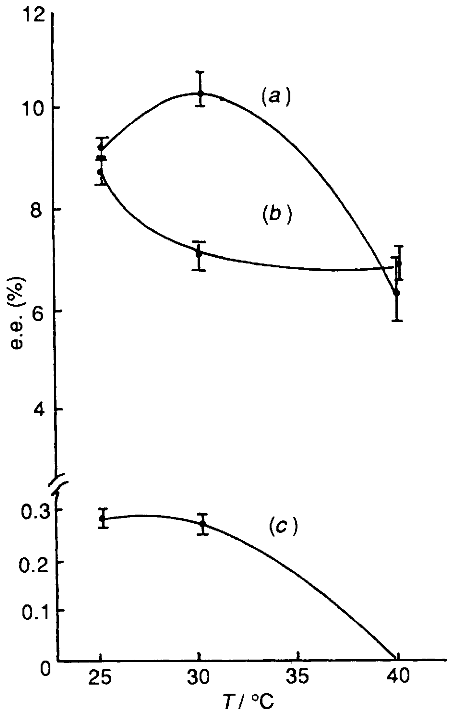

Fig. 4 Temperature effect on the stereoselectivity of the electron-transfer reaction at $I = 0.1$ mol dm^{-3} between cyt c(II) and (*a*) $[Co(bipy)_3]^{3+}$ in Tris-cacodylic acid buffer (pH 6.7), (*b*) $[Co(bipy)_3]^{3+}$ in phosphate buffer (pH 7.0) and (*c*) $[Co(pd)_3]$ in phosphate buffer (pH 7.0)

Discussion

Differences between $[Co(pd)_3]$ *and* $[Co(bipy)_3]^{3+}$.—There are several differences between these two cobalt(III) complexes. The complex $[Co(pd)_3]$ is neutral and hydrophobic, whereas the pd$^-$ ligand itself is hydrophilic.* In contrast, $[Co(bipy)_3]^{3+}$ is positively charged and hydrophilic, but its bipy ligand is hydrophobic and π-conjugated. As is well known, an active site of cyt c(II) is a haem edge which exists in a hydrophobic crevice and is partially exposed to solvent.[1a,2d,e,13] Electron transfer proceeds rapidly when a substrate can approach closely to the haem edge. In the case of $[Co(bipy)_3]^{3+}$ a hydrophobic bipy ligand can easily be incorporated into the crevice,[2d,f,g,14,15] which allows $[Co(bipy)_3]^{3+}$ to approach closely to the active site. This results in rapid reduction of $[Co(bipy)_3]^{3+}$. Hydrophobic interaction is considered to be important in such bipy incorporation into the crevice. In the case of $[Co(pd)_3]$, however, the pd$^-$ ligand is hydrophilic and its incorporation into the crevice is not easy. Therefore, close approach of $[Co(pd)_3]$ to the active site is difficult, and suppresses its reduction. Higher stereoselectivity in the reduction of $[Co(bipy)_3]^{3+}$ than of $[Co(pd)_3]$ would be also related to the incorporation of bipy into the crevice. The complex $[Co(pd)_3]$ would react with cyt c(II) at a long distance from the haem edge and the chiral groups existing around it, owing to lack of pd$^-$ penetration into the haem crevice. This results in small stereoselectivity for $[Co(pd)_3]$ reduction. In the case of $[Co(bipy)_3]^{3+}$, the incorporation of bipy into the crevice would allow closer contact of $[Co(bipy)_3]^{3+}$ with chiral groups existing around the crevice, which yields a greater stereoselectivity for this reaction.

The above-mentioned difference between $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$ is also a reason that they exhibit different dependences of the stereoselectivity on the temperature. For $[Co(pd)_3]$ the stereoselectivity disappears at 40 °C, as shown in Fig. 4. Because $[Co(pd)_3]$ cannot penetrate deeply into the crevice, the stereoselectivity would be mainly determined by chiral groups existing on the surface of cyt c(II). Absence of stereoselectivity at 40 °C, therefore, suggests that these chiral groups undergo vigorous thermal motion around 40 °C. Thermal motion of cyt c(II) has been investigated by the NMR technique by Moore and Williams.[16] Their experiment indicates that rapid tumbling of cyt c(II) is absent at < 37 °C but at > 57 °C rapid tumbling is enabled. This means that such thermal tumbling, which would accompany, at least, motion of amino acid residues existing on the surface, starts at around 40 °C (motion of internal amino amide residues is much more difficult, as described later). This temperature corresponds well with that at which stereoselectivity disappears.

For $[Co(bipy)_3]^{3+}$, on the other hand, moderately large

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* The negative charge of pd$^-$ is reduced by co-ordination to Co^{III}, and thereby, the hydrophilicity of pd$^-$ in $[Co(pd)_3]$ would be less than that of free pd$^-$. However, the pd$^-$ ligand in $[Co(pd)_3]$ is considered to be still hydrophilic because pentane-2,4-dione is considerably water-soluble in spite of its neutrality.[12]

stereoselectivity is observed even at 40 °C, which suggests that the chiral groups determining the stereoselectivity of $[Co(bipy)_3]^{3+}$ reduction differ from those determining the stereoselectivity of $[Co(pd)_3]$ reduction. Because the bipy ligand can penetrate deeply into the crevice (see above) the stereoselectivity of this reduction would be determined by chiral groups existing at relatively deep positions. The thermal motion of such chiral groups is considered to be difficult because they are fixed by hydrogen bonds. Thus, the stereoselectivity of $[Co(bipy)_3]^{3+}$ reduction is still observed at 40 °C. Again, an NMR experiment by Moore and Williams[15] supports our discussion; this revealed that motion of Phe-82, Phe-46 and Tyr-48 residues, which are all close to the haem group, is restricted even at rather high temperature, while motion of the Phe-36 and Tyr-74 existing at the surface is not noticeably restricted.

The stereoselectivity is much influenced by the buffer, and in quite different ways for $[Co(bipy)_3]^{3+}$ and $[Co(pd)_3]$, as described above. This is interpreted in terms of anion binding with cyt c; phosphate anion binds to the positively charged lysine residue of cyt c surrounding the solvent-exposed haem edge,* but the corresponding Tris–cacodylic acid anion cannot bind to cyt c. The binding of phosphate anion would weaken hydrogen bonds in cyt c(II), making cyt c(II) more flexible and probably facilitating crevice opening. This situation favours close approach of $[Co(pd)_3]$ to the chiral groups of cyt c(II). On the other hand, $[Co(bipy)_3]^{3+}$ can approach the chiral groups closely, even when the crevice is closed. Thus, phosphate binding is not necessary for the stereoselective reaction of $[Co(bipy)_3]^{3+}$ but is indispensable for the stereoselective reaction of $[Co(pd)_3]$. The phosphate binding also allows cyt c(II) to undergo thermal motion at a rather low temperature by weakening its hydrogen bonds. Thus, the stereoselectivity of $[Co(bipy)_3]^{3+}$ reduction decreases at 30 °C in the phosphate buffer. In Tris–cacodylic acid buffer, however, thermal motion is difficult at low temperature because of the absence of anion binding, and does not cause a decrease in the stereoselectivity at 30 °C. A similar discussion has been presented by Koller and Hawkridge[18] to explain the temperature effect on the electron-transfer reaction between cyt c and an indium oxide electrode. In their experiment the electron-transfer rate depends on the temperature and reaches its maximum at 41 °C in phosphate buffer but at 55 °C in Tris–cacodylic acid buffer, which has been interpreted in terms of phosphate binding with cyt c.

Dependence of Stereoselectivity on Ethanol Content in the Solvent.—In the case of $[Co(pd)_3]$ reduction, the stereoselectivity increases monotonously upon increasing the ethanol content. Because the pd^- ligand is not easily incorporated into a hydrophobic crevice, $[Co(pd)_3]$ reduction occurs at a rather long distance from the active site (see above). An increase in the ethanol content decreases the solvent hydrophilicity and would facilitate opening of the hydrophobic crevice. At the same time, a decrease in hydrophilicity enhances ion-pair formation between phosphate anion and the amino acid residues, which also facilitates crevice opening by weakening hydrogen bonds (see above). Such crevice opening would enhance approach of $[Co(pd)_3]$ to both the active site and chiral groups existing in the crevice, so increasing the stereoselectivity and accelerating electron transfer. This discussion is supported by the fact that the electron-transfer constant, k_{et}, increases with increasing ethanol content, as shown in Table 2.†

On the other hand, the $[Co(bipy)_3]^{3+}$ reduction exhibits a complicated dependence of the stereoselectivity on the ethanol content. Also, the kinetic parameters for the two complexes exhibit different dependences on the ethanol content. For $[Co(bipy)_3]^{3+}$ both k_{et} and K_M decrease with increasing ethanol content, which is opposite to the case with $[Co(pd)_3]$. An increase in ethanol content decreases the hydrophilicity in the bulk solution, which weakens the bipy incorporation into the crevice because this is caused by hydrophobic interaction. Thus, an increase in ethanol content suppresses the approach of $[Co(bipy)_3]^{3+}$ to the active site and slows down the electron-transfer step (see k_{et} values given in Table 2). Contrary to the decrease in k_{et}, the stereoselectivity slightly increases at 10% ethanol. An increase in ethanol content makes the structure of cyt c(II) flexible (see above), which would enhance close approach of chiral groups to $[Co(bipy)_3]^{3+}$. This favourable situation would be a reason for the slight increase in stereoselectivity. At higher ethanol content, however, the hydrophilicity of the bulk solution further decreases and the crevice opens too much. Under this condition, the bipy incorporation into the crevice is further weakened, which disfavours close approach of $[Co(bipy)_3]^{3+}$ to the chiral groups. At 20% ethanol this unfavourable condition would outweigh the favourable one mentioned above, and the stereoselectivity becomes small. In Tris–cacodylic acid buffer this unfavourable condition is unaffected by anion binding because Tris–cacodylic anion cannot bind with cyt c(II), and the stereoselectivity becomes smaller and smaller with increase in ethanol content, as shown in Fig. 4. In the phosphate buffer, however, the situation is changed by phosphate binding. If the crevice opens sufficiently, the phosphate anion can enter into it and bind with positively charged amino acid residues of cyt c(II). Such phosphate binding neutralizes the positively charged chiral groups and allows them to approach close to the positively charged $[Co(bipy)_3]^{3+}$. This would be a reason for the stereoselectivity increasing again upon going from 20 to 40% ethanol in the phosphate buffer.

Effect of pH *on the Stereoselectivity.*—The effect of pH on the stereoselectivity is not complicated like the effect of the ethanol content, as shown in Fig. 3. However, an interesting point is found. The stereoselectivity increases upon lowering the pH both for $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$, while the effects of pH on the kinetic parameters, k_{et} and K_M, are different for $[Co(pd)_3]$ and $[Co(bipy)_3]^{3+}$; with the former they increase upon lowering the pH, but for the latter they decrease.

As is well known, cyt c(II) adopts several conformational states depending on the pH. In the range pH 4.2–12.7 (27 °C) cyt c(II) is in the state II.[16,19,20] Thus, cyt c(II) adopts the same conformational state throughout almost all the pH range examined here, while the conformational state starts to change from II to I at pH 4.2. Nevertheless, the stereoselectivity and kinetic parameters change on lowering the pH from 7 to 4. This means that the kinetic parameters and stereoselectivity are more sensitive to the reaction conditions than is the conformational change. According to Moore and Williams,[16] cyt c(II) exhibits a NMR chemical shift for His-33 very similar to that of cyt c(III), and the pK_a of His-33 in cyt c(III) has been reported to be about 6.5.[21] Besides His-33, amino acid residues close to Ile-57 are reasonably supposed to undergo protonation around pH 5 because the chemical shift of Ile-57 starts to change around pH 5. Thus, protonation of His-33 and some amino acid residue close to Ile-57 must be taken into consideration here. Both amino acid residues exist at the surface rather distant from the exposed haem edge.[22] Such protonation, therefore, would not directly influence the electron-transfer reaction between cyt c(II) and cobalt(III) complexes, but weakens the hydrogen bonds of those amino acid residues and makes the structure of cyt c(II) more flexible. This means that the crevice is easy to open at low pH. The crevice opening would enhance close approach of $[Co(pd)_3]$ to the chiral groups, which favours the

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* Phosphate anion binding has been reported for cyt c(III).[17] The corresponding binding to cyt c(II) is also suggested by the experimental result that phosphate anion decreases the positive charge product of cyt c(II) and cobalt(III) complexes.[2j]

† K_M depends on k_1, k_{-1} and k_2. If k_2 is much smaller than k_{-1}, K_M corresponds to the reverse of the equilibrium constant for formation of [cyt c(II) $\cdots$ Co^{III}]. Because the values of k_2 and k_{-1} are unknown at the present we don't discuss the result, based on K_M.

Table 2 Dependence of Michaelis–Menten parameters on ethanol content * in the reduction of the cobalt(III) complexes

	Phosphate buffer		Tris–cacodylic acid buffer	
Ethanol (% v/v)	k_{et}/dm^3 mol^{-1} s^{-1}	K_M/mol dm^{-3}	k_{et}/dm^3 mol^{-1} s^{-1}	K_M/mol dm^{-3}
(*a*) $[Co(pd)_3]$ reduction				
15	1×10^{-4}	5×10^{-3}	1×10^{-4}	9×10^{-3}
20	2×10^{-4}	6×10^{-3}		
25	4×10^{-4}	26×10^{-3}	2×10^{-4}	11×10^{-3}
(*b*) $[Co(bipy)_3]^{3+}$ reduction				
0	28	89×10^{-3}	1.9	1.2×10^{-2}
10	8	15×10^{-3}	2.2	9×10^{-3}
15	5	7×10^{-3}		
20	3	3×10^{-3}	1.3	3×10^{-3}
25	3	2×10^{-3}	1.2	2×10^{-3}

* pH 7.0, $I = 0.1$ mol dm^{-3} (KCl).

Table 3 Effect of pH on Michaelis–Menten parameters in the electron-transfer reaction between cyt c(II) and the cobalt(III) complexes

	$[Co(pd)_3]^b$		$[Co(bipy)_3]^{3+\,c}$	
pH^a	k_{et}/dm^3 mol^{-1} s^{-1}	K_M/mol dm^{-3}	k_{et}/dm^3 mol^{-1} s^{-1}	K_M/mol dm^{-3}
4.0	14×10^{-4}	53×10^{-3}	10	25
6.0	1×10^{-4}	5×10^{-3}	16	62
7.0	1×10^{-4}	6×10^{-3}	28	89

[a] Acetate buffer was used for pH 4, and phosphate buffer for pH 6 and 7. [b] 15% Ethanol, $I = 0.1$ mol dm^{-3} (KCl). [c] 0% Ethanol, $I = 0.1$ mol dm^{-3} (KCl).

stereoselectivity. This would be one of the reasons why the stereoselectivity increases with lowering of pH.

Another factor would be more important in $[Co(bipy)_3]^{3+}$ reduction. In the study of the pH effect, phosphate and acetate buffers were used and both anions are considered to bind with cyt c(II). When the crevice opens at low pH, they bind with positively charged amino acid residues existing inside the crevice and neutralize their positive charge. Such charge neutralization allows those chiral groups to approach closely to $[Co(bipy)_3]^{3+}$, which would increase the stereoselectivity of $[Co(bipy)_3]^{3+}$ reduction at low pH.

Finally, we consider the pH effect on the reaction rate. The complex $[Co(pd)_3]$ can approach the active site only when the crevice is open, because the pd$^-$ ligand cannot easily penetrate the crevice. Lowering of the pH facilitates opening of the crevice (see above). This would be a reason why the electron-transfer step (k_{et}) is accelerated by pH lowering in the reduction of $[Co(pd)_3]$ (see Table 3). In the reduction of $[Co(bipy)_3]^{3+}$, penetration of bipy into the crevice allows $[Co(bipy)_3]^{3+}$ to approach the active site even when the crevice is closed. However, the more the crevice opens, the more the bipy penetration into the crevice is weakened because the hydrophobicity of the inside of the crevice decreases upon its opening (note hydrophobic interaction causes the bipy penetration). Thus, the electron-transfer step (k_{et}) becomes slower upon lowering of pH in the $[Co(bipy)_3]^{3+}$ reduction (see Table 3).

In conclusion, the reduction of $[Co(bipy)_3]^{3+}$ exhibits a much larger stereoselectivity and much more rapid electron-transfer reaction rate than does that of $[Co(pd)_3]$. Interesting dependences of the stereoselectivity and kinetic parameters on the reaction conditions were found. Furthermore, it is noted that the dependences are different for $[Co(bipy)_3]^{3+}$ and $[Co(pd)_3]$. All these results are successfully interpreted by considering that the hydrophobic bipy ligand can penetrate into the crevice but the hydrophilic pd$^-$ ligand cannot. This kind of stereoselective electron-transfer reaction of metalloenzymes would be expected to offer new information about the enzyme function, and further investigations are in progress.

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